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ISOLATION AND CHARACTERIZATION OF SOMATIC AND METABOLIC ANTIGEN--ETC(U)
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(6) Isolation and characterization of somatic and metabolic antigens and moulting fluid antigens of Dirofilaria immitis adults, microfilaria, late first stage (sausage-form) and third stage larvae.

(9) Final Comprehensive Report.

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(10) Stephen G. Zam

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Dirofilaria immitis adult, cuticular and microfilarial antigens have been obtained by a combination of phosphate buffer, 1% Triton X-100, 12% Triton X-100, butanol extractions and papain digestion. A number of adult, cuticular, and microfilarial antigens have been isolated by affinity chromatography. Antigen protein heterogenicity has been monitored by isoelectric focusing electrophoresis in polyacrylamide gels employing a pH gradient of 3-10. A protein-antigen coated polyacrylamide microbead agglutination test

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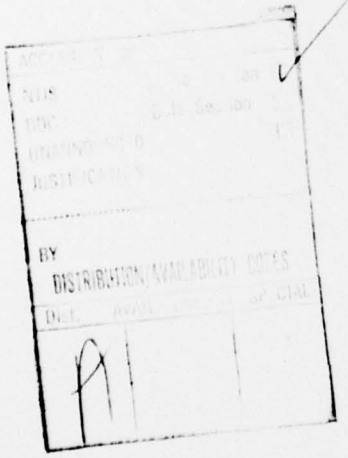
20. by antibody has been developed. Naturally infected dog sera in some cases in dilutions of up to 1:10,000 have agglutinated protein-antigen coated polyacrylamide microbeads. Absorption of sera antibodies by affinity chromatography serving as controls failed to agglutinate the microspheres. Antigens have been partially characterized by isoelectric focusing electrophoresis in polyacrylamide gels. Electrophoretically separated gels at pH 3-10, pH 3-6, pH 5-8 and pH 7-10 have been sectioned into 2 mm wafers. Eluted proteins were assayed fluorometrically for total protein content and by soluble antigen fluorescent antibody for antigens.



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Summary Statement of Final Comprehensive Report

The technical objectives of the investigation were: (1) the isolation, characterization and comparison of proteins, somatic, metabolic and moulting fluid antigens of four stages in the life cycle of Dirofilaria immitis; (2) the possible isolation of specific antigens of the four stages; (3) the determination of isolated antigen cross reactivity with other parasites; (4) determination of possible antigens excreted during the normal metabolism and moulting of the larval stages; (5) the possible development of a more specific skin test antigen for filarial infections.

Adult D. immitis were obtained from dogs at necropsy. Microfilariae were obtained from whole dog blood by a combination of blood clotting, erythrocyte lysis, pronase and tryptic digestion. Portions of isolated microfilariae served as antigen source. Other isolated microfilariae are being maintained by tissue culture in a fortified NCTC medium. Adult cuticular antigens have been obtained by 12% Triton X-100 and Butanol extractions followed by papain digestion of isolated cuticles.

Antibody obtained from naturally infected dogs has been tested by Soluble Antigen Fluorescent Antibody (SAFA), by heterocytotropic anaphylatic activity in rabbits, and by agglutination of protein antigen coated polyacrylamide microbeads (2 $\pm$ 1 micron).

A number of adult cuticular, adult buffer soluble and microfilarial antigens have been isolated by affinity chromatography. Precipitated dog gamma globulin coupled to activated Sepharose has served as the immunoabsorbent. Antigen and protein heterogenicity has been monitored by isoelectric focusing electrophoresis in polyacrylamide gels (IFPAG) employing a pH gradient 3-10. Adult antigens separated by preparative isoelectric focusing electrophoresis have been tested by SAFA and heterocytotropic anaphylatic activity in rabbits. Four adult protein fractions having molecular weights of: greater than 300,000, 100,000 to 300,000, 30,000 to 100,000 and 10,000 to 30,000 have been obtained by differential pressure ultrafiltration. Gel filtration on these fractions followed by narrow pH range isoelectric focusing electrophoresis has been employed. The nature of these protein fractions, molecular weights, types of protein, antigenicity, etc. are now being investigated.

The protein-antigen spectra of adult and microfilaria have been demonstrated by isoelectric focusing electrophoresis at pH 3-10, pH 3-6, pH 5-8 and pH 7-10. Electrophoretically separated gels have been sectioned into 2 mm wafers. Each wafer has been assayed for total protein fluorometrically using fluorescamine, for antigens by soluble antigen fluorescent antibody and pH profiles determined.

Adult and microfilarial buffer soluble and buffered 1% Triton X-100 soluble pooled protein homogenates were bound to aminoethyl and succinylated aminoethyl polyacrylamid microbeads (2 $\pm$ 1) microm) with a water soluble

carbodiimide, 1-ethyl-3(3dimethylaminopropyl) carbodiimide hydrochloride at pH 4.5 for 16 hours. The protein antigen bound microbeads were incubated with naturally infected dog sera at various dilutions. Of the dog sera tested, 92% gave positive agglutination of the acrylamide microbeads. In some instances serum dilutions of up to 1:10,000 gave positive results. No agglutination of the microbeads was observed in 96% of the controls. Although the specificity of this agglutination reaction has not been tested, this microbead agglutination test appears to be an excellent immunodiagnostic procedure for testing the presence of D. immitis antibodies. Protein-antigen bound microbeads after storage of 3 months at 4°C show agglutination in the presence of sera with little loss in sensitivity. The ease of protein-antigen-microbead coupling and the long shelf life of the coupled microbead hold promise as a new sensitive agglutination test for filarial infections.

Foreword:

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Science-National Research Council.

FINAL COMPREHENSIVE REPORT ON GRANT DAMD17-75-C-5009

I. PURPOSE OF INVESTIGATION

The technical objectives of the investigation were: (1) the isolation, characterization and comparison of proteins, somatic, metabolic and moulting fluid antigens of four stages in the life cycle of Dirofilaria immitis; (2) the possible isolation of specific antigens of the four stages; (3) the determination of isolated antigen cross reactivity with other parasites; (4) determination of possible antigens excreted during the normal metabolism and molting of the larval stages; (5) the possible development of a more specific skin test antigen for filarial infections.

II. PROGRESS REPORT

A. ISOLATION OF D. IMMITIS AND MICROFILARIAE

1. Adults:

Adults were obtained from the pulmonary vessels and right auricle of the heart by necropsy. Dogs used at the J. Hillis Miller Health Center, University of Florida were used as a source for all biological material. Immediately upon arrival at the laboratory, the adults were freed of clotted blood, rinsed five times in phosphate buffered saline (PBS) and processed for somatic antigen. Somatic antigen was stored at -75°C.

2. Microfilariae:

Units of freshly drawn dog blood with high microfilaremia (above 20,000 microfilariae/ml) were obtained from the jugular vein. On arrival at the laboratory protamine sulphate (0.5 gms/unit) was added to the blood. The blood was poured over two thirds of large petri dishes and left undisturbed for three hours at room temperature. The petri dish was tilted slightly and allowed to stand overnight. Serum containing microfilariae was collected from the bloodless portion of the petri dish. The collected serum was treated with twenty volumes of 0.2% Saponin at 38°C for 30 minutes to lyse the erythrocytes. Microfilariae, redblood cell ghosts and leucocytes were collected by using a Szent-Gyorgyi and Blum continuous flow centrifugation system. The recovered microfilariae were then treated with pronase, followed by tryptic digestion and washed in saline. Periodically small clumps or strands of material were observed in the final preparation that could not be dispersed by enzymatic treatment. Isolated microfilariae were stored at -75°C.

B. ANTIGEN PREPARATION:

1. Buffer soluble adult antigen

The worms were minced in iced (4°C) 0.05M sodium phosphate buffer, pH 7.4 and homogenized in a Waring blender. The resulting homogenate was further homogenized in an iced TenBroeck tissue homogenizer. The homogenate was extracted overnight at 4°C. The precipitate was extracted overnight in 1% Triton X-100 at 4°C. The Triton X-100 extract was treated with Bio-Beads Sm-2 to remove the Triton. The phosphate buffered and Triton X-100 extracts were pooled and used as adult somatic antigen.

2. Adult cuticular antigen

The 1% Triton X-100 precipitate was extracted overnight in 12% Triton X-100 at 4°C. After extraction and centrifugation, the supernate was retained. The 12% Triton X-100 precipitate was extracted for 30 minutes with iced n-butanol. The homogenate was centrifuged and the aqueous phase retained. The butanol extracted homogenate was washed repeatedly to remove traces of butanol. This precipitate was digested with enzide papain (papain attached to polyacrylamide) for 30 minutes. The homogenate was centrifuged and the supernate retained. Protein was found in the butanol papain extract but not in the 12% Triton X-100 extract.

3. Microfilariae

Isolated microfilariae in iced PBS were sonicated until microscopic examination showed no intact microfilariae. Microfilarial somatic antigen was prepared as in the procedure for buffer soluble adult antigen.

4. Dog blood debris

Dog blood free of microfilariae was clotted and the saline washed clot extracted with saponin, pronase and trypsin similar to microfilarial isolation. A quantity of fibrous contaminant was obtained identical to the contaminant periodically present in the microfilarial purification procedure. This contaminant was sonicated, extracted overnight, centrifuged and the debris treated with Triton X-100 as described previously.

C. ANTIBODY PREPARATION:

1. Dog

Sera from naturally infected dogs having high microfilaremia was harvested. The dog sera was precipitated three times at one-half ammonia sulphate saturation, pH 6.8. The final precipitate was exhaustively dialyzed against 0.1 M $NaHCO_3$, pH 6.8.

2. Rabbit

Male New Zealand rabbits were immunized with D. immitis adult, microfilariae and dog blood debris for 5 weeks. The immunogen contained 6-10 mg of protein emulsified with equal amounts of Freund's complete adjuvant injected intramuscularily. Rabbits were bled by cardiac puncture and sera obtained. Rabbit sera was precipitated with ammonia sulphate at final concentration of one-third saturation. The precipitate was dissolved in saline and exhaustively dialyzed against 0.1 M $NaHCO_3$, pH 6.8.

D. ANTIGEN-ANTIBODY ASSAY

1. Soluble antigen fluorescent antibody (SAFA)

Dog sera and antigenic homogenates were assayed by the standard SAFA procedure. Dilution of antigen or antibody were 1:1, 1:2, 1:4, 1:8, 1:16.

2. Heterocytotropic Anaphylactic Activity in Rabbits

Sera from dogs were tested on rabbits with 48 to 72 hour latency. Serial dilutations of sera were injected intradermally. The challenging parasitic antigen was 0.4 mg protein in 0.5% Evans Blue or potamine sky blue solution per kg of body weight. Blue spots with a diameter 5mm or greater than those of the control were regarded as positive reactions.

3. Agglutination of protein-antigen coupled polyacrylamide microbeads by D. immitis antibody.

Adult and microfilarial antigens (2.8-4.2 mg) were coupled to aminoethyl Affi-gel 701 and succinylated aminoethyl Affi-gel 702 (BioRad Laboratories) polyacrylamide microbeads (2 $\pm$ 1 micron) using a water soluble carbodiimide EDAC, 1-ethyl-3(3dimethylaminopropyl) carbodiimide hydrochloride. All solutions used in the coupling procedures were filtered through 0.45 um millipore filters prior to use. On addition of the carbodiimide, the pH was maintained at pH 4.8 for 16 hours at 4°C. After coupling the gels were exhaustively washed in 0.01 M barbital buffer, pH 7.4 to remove uncoupled protein and EDAC. Dog sera from naturally infected dogs were filtered through 0.45 um millipore filters. Sequential sera dilutions were made with 0.01 M barbital buffered, pH 7.4, 0.08 M NaCl. An aliquot (0.05 ml) of the diluted sera was added to the wells (V shaped) of a plastic disposable hemagglutination tray. Aliquots (0.05 ml) of protein antigen coupled polyacrylamide microbeads were added, and the tray contents allowed to incubate at room temperature for 3 to 5 hours. After incubation the tray was vibrated on a rotary

platform shaker for 2 mn. The vibration caused the agglutinated polyacrylamide spheres to settle in the "V" shaped bottom of the well. Of the 25 dogs tested having microfilaremias of 10,000 - 40,000 microfilariae/cc clear end points were observed on 92% of the sera tested. A clear end point was observed on sera up to dilutions of 1:200. Some sera (20%) showed clear end points at 1:1,000 dilutions with a small percentage (12%) showing gel agglutination at 1:10,000 sera dilutions. The sensitivity of the microbead agglutinization test varied with the type of acrylamide microbeads and antigen homogenate employed. The clearest end points and highest sera dilution titers were observed with Affi-gel 70i coupled adult homogenates. Protein-antigen coupled beads stored for 3 months at 4°C produced agglutination in the presence of antisera.

E. ANTIGEN SOLUTION

1. Affinity Chromatography

Ammonium sulphate precipitated dog immunoglobulins were coupled to Affi-gel 10 (Bio-Rad Laboratories), a n-hydroxy-succinimide ester of succinylated aminoalkyl Bio-Gel A support, an agarose gel matrix. Twenty-five ml of dog immunoglobulin, pH 6.8 was shaken with 3 gm Affi-gel 10 overnight. A 2 cm x 45 cm column was poured and extensively washed with 0.1 M sodium phosphate buffered 0.15 M NaCl, pH 7.4. Dirofilaria adult, microfilarial and cuticular homogenate were applied to the column and eluted with wash buffer at a flow rate of 10 ml/hr. After the elution of non specific protein was completed, the column was washed with 0.1 M HCl, 1.0 M HCl and 2 M HCl at an elution rate of 30 ml/hr. Some columns were also eluted with 0.1 M glycine-HCl buffer, pH 3.2 and 0.1 M glycine-HCl buffer, pH 2.3. Eluted fractions were monitored at 280 mu and neutralized with NaOH. Dog blood cell debris was bound to 1 gm of Affi-gel 10 as described above. Immunodiffusion and immunoelectrophoresis shows the dog blood debris contaminant exhibits a common precipitant band when tested with rabbit antisera. This contaminant was absorbed out of microfilaria homogenated by batch absorption onto Affi-gel 10 coupled with rabbit antidebris immunoglobulin. Elution profiles of adult and microfilarial homogenates are similar. After elution of non absorbed proteins, proteins of both bound to the immunoabsorbent elute off with peaks at 0.1 M, 1.0 M and 2 M HCl. The highest peak of microfilariae elute at 1.0 M HCl, while the highest adult peak elute at 0.1 M HCl. These peaks show antigenicity when tested by SAFA. Nonabsorbed protein peaks eluted in wash buffer show no SAFA activity. When absorbed adult and microfilariae are eluted with 0.1 M glycine-HCl, pH 3.2 and 2.3 both fractions are antigenic when tested by SAFA. The highest peak being observed in both at pH 3.2. When butanol and papain extracted cuticle are chromatographed on Affi-gel 10 columns only two fractions, one at 1.0 M HCl and one at 2.0 M HCl are recovered.

2. Preparative isoelectric focusing electrophoresis

Adult homogenates have been electrophoresed on an LKB 440 preparative sucrose gradient isoelectric focusing electrophoresis pH 3-10. Fourteen major protein fractions separated through the full pH range have been isolated by electrophoresis at 300 volts for three days. These fractions are now being assayed for antigenicity and for number of proteins present. Microfilarial homogenates are now being electrophoresied. When completed, they will be assayed for antigenicity and number of proteins.

3. Differential ultrafiltration

Adult homogenates have been filtered through Amicron ultrafiltration membranes UM300, UM100, PM30 and PM10. The retentates have been washed with 10 volumes 0.05 M Tris-HCL, pH 7.4 buffered 0.15 M NaCl. Four protein fractions having molecular weights of: greater than 300,000, 100,000-300,000, 30,000-100,000 and 10,000-30,000 have been fractionated. These fractions are now being assayed by SAFA, heterocytotropic anaphylactic activity in rabbits, and by isoelectric focusing electrophoresis in polyacrylamide gels. When sufficient quantities of microfilariae are obtained they also shall be fractionated.

4. Gel filtration

Adult ultrafiltration retentate retained by filtration through Amicron ultrafiltration membrane UM 300 has been chromatographed on LKB ultragel AcA 22 (acrylamide/agarose gel separation range 60,000-1,000,000). Two protein peaks have been separated in the UM 300 retentate. The remaining retentates are being chromatographed on AcA 34 (separation range 20,000-400,000) and AcA 54 (separation range 6,000-70,000). When completed these fractions will be assayed for antigenicity and protein content. The columns are being calibrated with known molecular weight markers to ascertain the molecular weights of the various isolated Dirofilaria proteins.

5. Isoelectric focusing in polyacrylamide gels (IFPAG)

Isoelectric focusing electrophoresis in polyacrylamide gels (6%/3% acrylamide/bis-acrylamide), pH range 3-10 has been employed to measure protein homogenicity or heterogenicity. Gels have been stained with Bromophenol blue, Coomassie brilliant blue R-250, periodic acid - Schiff (PAS), Toludien blue and Alcian blue. Electrophoresis of adult and microfilarial homogenates show protein banding through the entire pH range with a bewildering number of proteins. Cuticular extracts of adult show proteins restricted to the basic pH range during electrophoretic separation. Five

positive PAS and four positive Alcian blue bands are present in the cuticular extracts indicating glycoprotein. No staining of cuticular protein was observed with Toludien blue.

Isoelectric focusing electrophoresis of adult and microfilarial homogenates have been performed using pH gradients of pH 3-10, pH 3-6, pH 5-8 and pH 7-10 to determine the number of somatic antigens. On completion of the electrophoresis each gel was frozen and sectioned into 2 mm thin wafers from anodal to cathodal end. The wafers were eluted with 0.01 M KCl and the pH determined. Ampholene was removed by the addition of a mixed bed resin, AG 501-X8. Protein determinations were made on each wafer fluorometrically using fluorescamine. Aliquots of eluted protein were assayed by SAFA. Twelve antigenic peaks have been demonstrated on adult homogenates and five antigenic peaks on microfilarial homogenates separated in the pH 3-10 range. The majority of the antigens show isoelectric points in the pH 3-6 range.

Conclusions:

A rapid sensitive agglutination test has been developed employing inert antigen coupled polyacrylamide microbeads. This assay shows some promise as an immunodiagnostic procedure. This microbead agglutination test overcomes the disadvantages of hemagglutination. Hemagglutination using sheep red blood cells as antigen carriers shows nonspecific agglutination and a drop in antibody titer during storage. The antigen coated microbeads overcome these disadvantages, have long shelf life, are easily coupled and are easily manipulated. Clear end points with high sensitivity are obtained in relatively short periods. Affinity chromatography and isoelectric focusing electrophoresis are excellent separatory techniques for D. immitis antigen isolations. Large quantities of antigens may be isolated using these techniques. Of interest seldomly investigated are the cuticular antigens. One major problem is solubilization of cuticular proteins. The role of these cuticular antigens in the host-parasite response merits further investigation.

Recommendations:

Further investigations on improving the polyacrylamide agglutination test would be beneficial. This test shows promise as a sensitive immunodiagnostic procedure for filarial infections. Although high sensitivity has been demonstrated, the specificity has not. Filarials cross react with a number of other helminths, viral and veneral diseases when tested with bentonite flocculation and hemagglutination. Further antigen purification prior to microbead coupling would seem warranted to improve specificity. Another possible approach would be to absorb out cross reacting antigens by specific cross reacting sera coupled to affinity chromatography gels.

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